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Phorbol ester-induced translocation of PKC epsilon to the nucleus in fibroblasts: identification of nuclear PKC epsilon-associating proteins

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Abstract We show that phorbol ester treatment of NIH 3T3 fibroblasts induces rapid translocation of PKCε from a perinuclear site to the nucleus, extending findings in PC12 and NG108-15 cells and in myocytes. We have immunoprecipitated the PKCε from nuclei isolated from phorbol ester-treated fibroblasts and identified six proteins which associate with nuclear PKCε. These have been characterised as matrin 3, transferrin, Rac GTPase activating protein 1, vimentin, β-actin and annexin II by MALDI–TOF–MS. We have confirmed that these proteins associate with PKCε by gel overlay and/or dot blotting assays. The role of these PKCε-associating proteins in the nucleus and their interaction with PKCε are considered.

Keywords: Protein kinase Cε; Nuclear translocation; Phorbol ester; Immunoprecipitation; MALDI–TOF–MS; Nuclear PKCε-binding protein

1. Introduction

Translocation of protein kinase C isoforms from the cytosol to the plasma membrane is a commonly observed effect following the stimulation of cells with a range of ligands including phorbol ester analogues of diacylglycerol (DAG) [1,2]. This translocation results in activation and turnover of the kinase and the phosphorylation of downstream substrates [3]. Translocation of PKC isoforms to cell compartments other than the plasma membrane has also been reported [4,5]. PKCε, for example, translocates from a perinuclear region to the cytosol on ethanol treatment of NG108-15 cells [6] while the anticancer agent *cis*-diamminedichloroplatinum induces translocation from the plasma membrane to the cytosol and nuclear membrane in SKBR-3 cells [7]. PKCε is the only fully oncogenic PKC isoform and its translocation to the nucleus occurs, for example, when NG108-15 cells are stimulated with phorbol ester, when myocytes are treated with arachidonic acid, when PC12 cells are treated with serum [6,8,9] or in ischaemic preconditioning [10]. Indeed, a role for PKCε translocation to the nucleus in cardioprotection has been suggested [11] but target substrates that PKCε associates with in the nucleus are not well defined. Here we show that phorbol ester treatment of 3T3 fibroblasts induces PKCε translocation to the nucleus. We have recovered six proteins which associate with nuclear PKCε and have identified them by MALDI-TOF mass spectrometry as being involved in regulating cell proliferation, apoptosis, DNA replication, mRNA processing and nucleoskeleton organisation.

2. Materials and methods

2.1. Cell culture and immunofluorescence

NIH3T3 cells (ECACC, Porton Down, UK) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (Gibco BRL, Paisley, UK) to about 80% confluency. For experiments, cells were passaged in fresh medium into 4-chamber culture slides or plastic dishes 24 h before treatments. Cells in 4-chamber culture slides (Nunc, Life Technologies, Paisley, UK) were treated with 1 μM PMA or DMSO vehicle for 30 min and PKCε was detected by immunofluorescence as described previously [12].

2.2. Construction of a plasmid encoding GFP-PKCε fusion protein and observation in living cells

A plasmid containing humanised GFP cDNA (pEGFP-C1) was obtained from Clontech (Palo Alto, USA). The PKCε encoding sequence was generated by PCR from a pGEM-PKCε plasmid [13]. The sense primer with a Bg1 II restriction site was 5′-GAGA-AGATCTATGGTAGTGTTCAATGGCCTTC-3′. The antisense primer with an *Eco*RI site was 5′-TCTCGAATTCTCAGGGCAT-CAGGTCTTCACC-3′. The insert was confirmed by sequencing. 3T3 cells were transfected with the GFP-PKCε plasmid using Polyfect Transfection Reagent (Qiagen) using the maker's protocol. Cells were treated with 1 μM PMA or DMSO 24 h after transfection. Translocation of GFP-PKCε was monitored under a Zeiss confocal laser scanning microscope at 37 °C in a hood supplied with 5% CO_2.

2.3. Isolation of nuclei

Nuclei were isolated as described by Dignam et al. [14] with modifications. Cells treated with PMA (30 min) or vehicle were scraped from dishes in cold PBS and pelleted by centrifugation at 700 × *g* for 5 min. Pelleted cells were resuspended in 5 vols hypotonic buffer (15 mM Tris–HCl, 15 mM NaCl, 60 mM KCl, 0.5 mM EDTA, pH 7.4, plus protease inhibitor cocktail) and left on ice for 15 min. Cells were then lysed with 0.5% Triton X-100 (final concentration) and passed several times through a 23-gauge needle. Nuclei were then pelleted at 1000 × *g* for 10 min. The pelleted nuclei were washed twice by resuspension in cold PBS and centrifuged at 25 000 × *g* for 20 min to remove residual cytoplasmic material. The purity of the nuclear fraction was examined by staining with 1 μg/ml DAPI for 15 min at 37 °C and observation

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Abbreviations: DAG, diacylglycerol; DIC, differential interference contrast; GFP, green fluorescent protein; MALDI–TOF–MS, matrix-assisted laser desorption/ionisation time of flight mass spectrometry; NGS, normal goat serum; NLS, nuclear localisation sequence; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Rac GAP1, Rac GTPase-activating protein 1; RACK, receptor for activated C kinase

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under a Zeiss confocal laser scanning microscope. To check the nuclear preparation for cytoplasmic contamination, aliquots of the supernatant above the nuclear pellets and of nuclear lysate were resolved by SDS–PAGE, followed by Western blotting with a monoclonal antibody (1:10 000) to detect α-tubulin.

2.4. Co-immunoprecipitation of proteins binding to nuclear PKCε and MALDI–TOF–MS analysis

Nuclei from PMA-treated cells were resuspended in immunoprecipitation buffer (50 mM Tris–HCl, 300 mM NaCl, 5 mM EDTA, 0.5% SDS, 1% Triton X-100, 1 mM PMSF, 2 μg/ml leupeptin, 1 mM AEBSF, pH 7.4) and passed several times through a 25-gauge needle. The nuclear lysate was centrifuged at $14\,000 \times g$ for 10 min to remove any insoluble proteins, analysed for protein content (BCA assay) and then precleared with protein G-sepharose (PGS) for 1 h. The precleared nuclear lysate was divided into two samples, each containing equal amounts of nuclear protein. One sample was then incubated with mouse anti-PKCε antibody (17 μg/ml in final concentration) and the other with mouse IgG for 2 h. Antibody complexes were recovered with PGS beads. After rinsing the beads three times with immunoprecipitation buffer, proteins were solubilised in Laemmli or thiourea buffer. Immunoprecipitates were resolved by SDS–PAGE (8–18% gels) or 2D electrophoresis and proteins located by silver staining. Specific bands or dots were excised into 1 mm blocks. After destaining, the blocks were dehydrated with acetonitrile. Samples were digested with trypsin (Promega, Madison, USA) overnight at 37 °C. A 1 μl aliquot of digestion supernatant was spotted onto a MALDI–TOF–MS sample plate with 0.6 μl matrix and allowed to air-dry. An Applied Biosystems 4700 Proteomics Analyser, coupled with tandem mass spectrometry (MS/MS), was used to generate peptide mass fingerprints. These were searched against the NCBInr public database. A mass fingerprint was considered a significant match if the score was equal to or greater than 75.

2.5. Gel overlay assay for PKCε-binding proteins in nuclei

Nuclear proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with 2 μg/ml recombinant PKCε (CalBiochem, Nottingham, UK) in 1% milk powder (Marvel) in TBS for 2 h followed by three rinses with TBS. Membranes were then processed for Western blotting with a PKCε antibody (1:200) as above. Controls were incubated with 1% milk powder lacking PKCε.

2.6. Dot blot assay

For dot blotting, purified transferrin, vimentin, β-actin and annexin II (Sigma) were applied directly to nitrocellulose membrane: as a control, an equal amount of BSA was applied. Protein spots were air dried and membranes were then blocked with 5% milk powder in TBS. The membranes were then incubated with 2 μg/ml recombinant PKCε in 1% milk powder in TBS overnight and rinsed three times with TBS. After washes, the membranes were processed for Western blotting with a PKCε antibody (1:200) as above.

3. Results

3.1. PKCε translocates to the nucleus upon PMA stimulation in fibroblasts

In normal fibroblasts PKCε has a mainly perinuclear localisation (Fig. 1A, CON). Treatment of cells with 1 μM PMA for 30 min induced an obvious translocation to the nucleus as revealed by the increased fluorescence associated with the nucleus and the decrease in PKCε localisation in the perinuclear region (Fig. 1A, PMA). To confirm this, we transfected NIH3T3 with GFP-PKCε and monitored the real time translocation of GFP-PKCε in living cells treated with 1 μM PMA by confocal microscopy for up to 60 min. In agreement with the immunofluorescence results, GFP-PKCε was localised in the perinuclear region before PMA stimulation (Fig. 1B, 0 m) but translocated to the nucleus on PMA stimulation. This real time translocation to the nucleus was especially obvious after 30 min (Fig. 1B, 30 m). To confirm this finding further, we isolated nuclei from control and PMA-treated fibroblasts and analysed them for PKCε by Western blotting. As shown in Fig. 1C, the PKCε band was more intense in nuclei of cells treated with PMA confirming that phorbol ester treatment of fibroblasts induces the nuclear translocation of PKCε.

3.2. Identification of nuclear PKCε-binding proteins in PMA-treated fibroblasts

We isolated nuclei from PMA-treated cells and coimmunoprecipitated PKCε-associating proteins for identification by MALDI–TOF–MS. The accuracy of such experiments depends on the purity of the nuclei so this was examined by DAPI staining and by Western blotting. Staining the nuclei preparation with DAPI matched the differential interference contrast (DIC) picture of a typical nuclei preparation with no obvious contamination by other organelles such as endoplas-

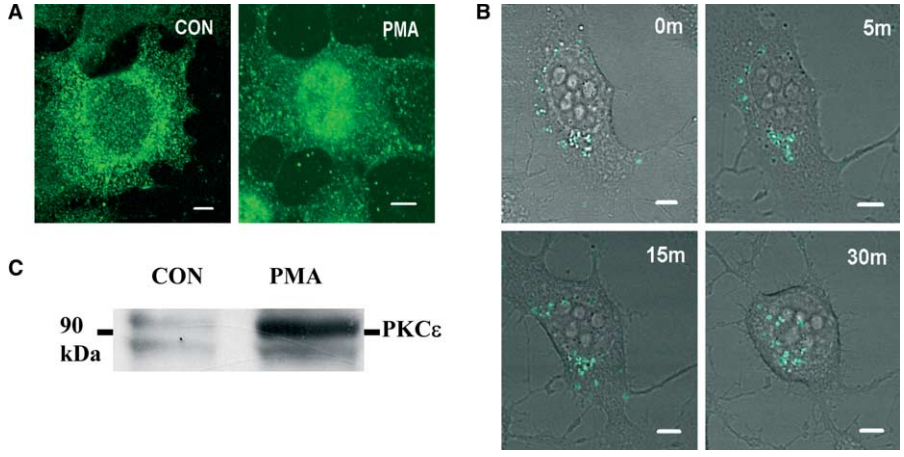

Fig. 1. PMA induces the nuclear translocation of PKCε in fibroblasts. (A) Immunofluorescence staining of PKCε in 3T3 cells. CON, PKCε is predominantly localised in the perinuclear region of cells treated with DMSO vehicle. PMA, PKCε in cells treated with 1 μM PMA for 30 min has translocated to nucleus. (B) Real time observation of GFP-PKCε in living 3T3 cells treated with 1 μM PMA. 0 m, on addition of PMA GFP-PKCε is localised in a perinuclear region; 5 m, after 5 min GFP-PKCε is still localised in perinuclear region; 15 m, after 15 min some GFP-PKCε moves into the nucleus; 30 m, after 30 min most GFP-PKCε has moved to nucleus. (C) Western blotting detection of PKCε in nuclei. CON, The level of PKCε in nuclei from DMSO vehicle treated cells is low. PMA, PKCε in nuclei from PMA treated cells is increased. Scale bar in (A) and (B) represents 5 μm.

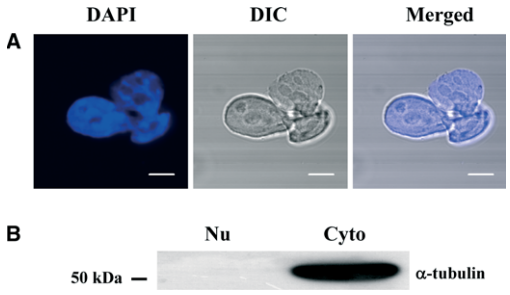

Fig. 2. Purity of nuclear preparations. (A) Purified nuclei were stained with the DNA binding dye DAPI and compared with the DIC image. The merged DAPI/DIC image suggests nuclei were highly purified with no evidence of contaminating organelles. (B) The cytoskeleton protein α-tubulin was not detected in the lysate of purified nuclei, but was present in the cytoplasmic fraction. Scale bar in (A) represents 5 μm.

mic reticulum (Fig. 2A). This was confirmed by Western blotting for a typical cytoplasmic/cytoskeletal protein, α-tubulin, which was not detected in the nuclear preparation (Fig. 2B).

To minimise the possibility of artificial binding of proteins to PGS beads, we precleared the nuclear lysate and used a mouse IgG control. Immunoprecipitates were separated on 8–18% gradient gels and only bands absent from or increased in concentration over IgG control immunoprecipitates were taken for MALDI–TOF–MS identification. We found that five proteins met such criteria (Fig. 3). With more precise 2D electrophoresis, another protein, annexin II, was also identified in PKCε immunoprecipitates (data not shown). The protein score in MALDI–TOF–MS, accession number in NCBInr and role in the nucleus for these six proteins are summarised in Table 1. Another three proteins were detected in PKCε immunoprecipitates compared with IgG control, but as we have not yet confirmed that these bind PKCε by gel overlay or dot blotting they are not included in Table 1.

3.3. Confirmation of PKCε-binding by gel overlay and dot blotting assays

We confirmed that the six proteins identified in PKCε coimmunoprecipitates associated with recombinant PKCε by gel overlay assays. As shown in Fig. 4A proteins associating with PKCε were detected at the expected molecular sizes for the six proteins identified by MALDI–TOF–MS (Table 1) compared with control blots not incubated with recombinant PKCε. Four of the nuclear proteins identified binding to PKCε, transferrin, vimentin, β-actin and annexin II are available commercially, so we verified that these proteins associated with PKCε by a dot blotting assay showing duplicate results

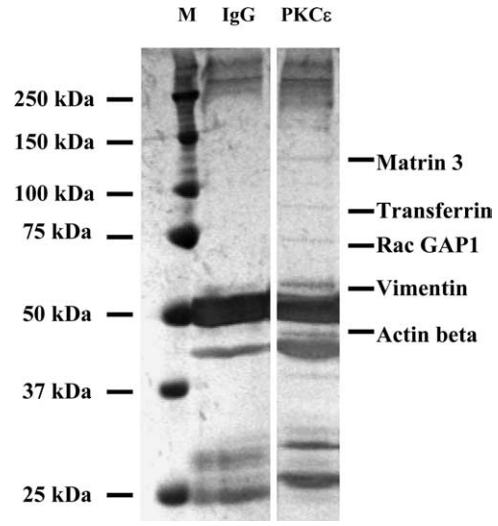

Fig. 3. Identification of PKCε coimmunoprecipitates on gradient gels. Proteins coimmunoprecipitating with nuclear PKCε were separated on 8–18% gradient gels and stained by MALDI–TOF–MS-compatible silver staining. Lane M, markers; Lane IgG, mouse IgG coimmunoprecipitate control; Lane PKCε, PKCε coimmunoprecipitate. By this approach 5 proteins, matrin 3 (125 kDa), transferrin (80 kDa), Rac GAP1 (70 kDa), vimentin (50 kDa) and β-actin (42 kDa) were identified.

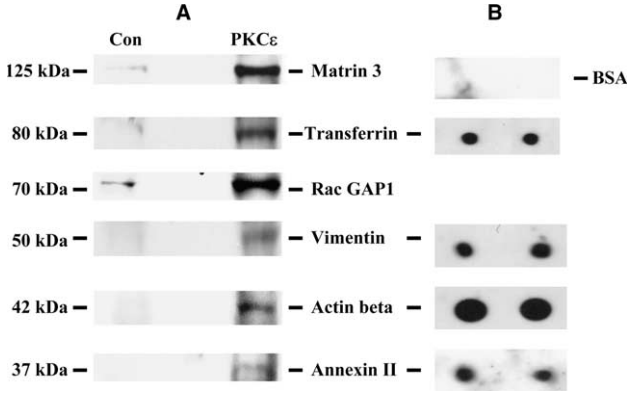

Fig. 4. Gel overlay and dot blotting. (A) Gel overlay assay. Results confirm that the six proteins identified in the nuclear PKCε immunoprecipitates bind recombinant PKCε compared with controls (Con). (B) Dot blotting assay. Results in duplicate confirm that transferrin, vimentin, β-actin and annexin II bind PKCε whereas BSA control does not.

Table 1
MALDI–TOF–MS identification of proteins associating with nuclear PKCε in 3T3 cells

Protein	MW (kDa)	Accession number	Protein score in MALDI–TOF–MS	Main role in nucleus
Matrin 3	93[a]	gi|25141233	458	RNA splicing
Transferrin	80	gi|21363012	112	Cell proliferation
Rac GAP1	70	gi|6755266	195	Cytokinesis
Vimentin	51	gi|2078001	186	Chromatin organisation
Actin beta	42	gi|71619	94	mRNA transcription
Annexin II	37	gi|71763	90	DNA replication

[a] Apparent molecular weight on SDS–PAGE is 125 kDa [24].

(Fig. 4B). Bovine serum albumin as a negative control did not bind PKCε (Fig. 4B).

4. Discussion

Translocation of PKC isoforms on cell stimulation occurs to activate the kinase, to bring the activated enzyme into association with downstream substrates and to initiate its inactivation [3]. PKC isoforms require a phosphatidylserine surface in membranes for activity but, for full activation, novel PKCε requires DAG generated in membranes by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by a phosphatidylinositol-phospholipase C. Cell stimulation can induce PKCε translocation to the plasma membrane [1], the Golgi apparatus [15], a perinuclear localisation [16], mitochondria [17] or the nuclear membrane and into the nucleus [4,6]. Nuclear translocation has been noted in PC12, NG108-15 cells and myocytes treated with serum, phorbol ester and arachidonic acid respectively and is also induced by ischaemic preconditioning which leads to cardioprotection [10]. Translocation of PKCε to the nucleus is of interest since this isoform has oncogenic potential and is implicated in cardioprotection [11]. It thus becomes important to identify the proteins interacting with PKCε after entry into the nucleus. We show here by three methods, including examination in living cells, that phorbol ester stimulation of fibroblasts induces PKCε translocation to the nucleus. This finding therefore extends the range of cells in which nuclear translocation of PKCε has been observed. Some PKCε is present in the nuclei of fibroblasts prior to PMA treatment as revealed by our immunofluorescence and Western blotting results but levels of nuclear PKCε clearly increase on PMA treatment. Nuclear PKCε may be in an activated state since DAG occurs within the nucleus [18,19]. Levels of nuclear DAG can alter during the cell cycle and generation of polyunsaturated DAG species correlates with PKC activation [20]. Tetra unsaturated species of DAG increase during the G_2/M phase of the cell cycle and lead to translocation of $PKC\beta_{II}$ to the nucleus at G_2/M [19]. How PMA added exogenously to fibroblasts causes PKCε movement to the nucleus is not clear though nuclei have receptors for PMA [21] while a cytosolic PMA-binding protein can function in HeLa cells to transport PMA to the nucleus [22]. The latest prediction software indicates that PKCε lacks a nuclear localisation sequence (NLS) in agreement with Wooeten et al. [23]. However, PKCε activated at the plasma membrane by PMA may be co-transported to the nucleus in association with a protein which has an NLS. Interestingly, matrin 3 which we have identified as a binding partner for PKCε in the nucleus, has a potential NLS [24]. Matrin 3, a component of the fibrogranular nucleoskeleton with eleven potential PKC phosphorylation sites (as identified using Phosphobase [25]) may therefore be a substrate of PKCε. We are now investigating whether phosphorylation of matrin 3 by PKCε enhances movement of a kinase-matrin 3 complex to the nucleus where transcription and RNA splicing, known functions of matrin 3 [24,26,27], would be influenced.

Four of the five other proteins we have identified associating with PKCε in the nucleus, namely vimentin, transferrin, annexin II and RacGAP1 are regulated by phosphorylation which may explain their association with PKCε. Vimentin, involved in chromatin organisation and nuclear matrix core filament construction [28], has eleven PKC phosphorylation sites [25] while iron-binding transferrin, which can bind to DNA with strong affinity, has seven [25]. RacGAP1 mainly localises in the nucleus in interphase, accumulates on the mitotic spindle in metaphase and condenses in the midbody [29]. Phosphorylation at Ser387 is important for the cytokinesis function of RacGAP1. Aurora B might be responsible for this phosphorylation [30] but our results suggest that PKCε in the nucleus might also influence the function of RacGAP1. Nuclear annexin II, implicated in DNA replication and cell proliferation [31,32], is also regulated by phosphorylation at Ser11 and Ser25 in the nuclear export signal domain [32].

Actin is a normal component of the nucleus involved in mRNA transcription and nuclear assembly [33,34]. It is not surprising that we detected actin associated with nuclear PKCε since this isoform has a unique actin binding motif and the association of actin with PKCε can maintain the kinase in a catalytically-active conformation [35].

A role for PKCε translocation to the nucleus in protection against ischaemic injury [11] has been identified, making it essential to identify the precise pathways by which this event protects against myocardial ischaemia. This will be achieved when the proteins that PKCε associates with in the nucleus are defined, determining which are substrates and which are receptors for activated C-kinase (RACKS) localising PKCε to specific nuclear sites.

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